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Prenatal exposure to bisphenol A promotes angiogenesis and alters steroid-mediated responses in the mammary glands of cycling rats

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ABSTRACT

Prenatal exposure to BPA disturbs mammary gland histoarchitecture and increases the carcinogenic susceptibility to chemical challenges administered long after BPA exposure. Our aim was to assess the effect of prenatal BPA exposure on mammary gland angiogenesis and steroid hormone pathways in virgin cycling rats. Pregnant Wistar rats were exposed to either 25 or 250 μg/kg/day (25 and 250 BPA, respectively) or to vehicle. Female offspring were autopsied on postnatal day (PND) 50 or 110. Ovarian steroid serum levels, the expression of steroid receptors and their co-regulators SRC-3 and SMRT in the mammary gland, and angiogenesis were evaluated. At PND 50, all BPA-treated animals had lower serum levels of progesterone, while estradiol levels remained unchanged. The higher dose of BPA increased mammary ERα and decreased SRC-3 expression at PND 50 and PND 110. SMRT protein levels were similar among groups at PND 50, whereas at PND 110, animals exposed to 250 BPA showed a lower SMRT expression. Interestingly, in the control and 25 BPA groups, SMRT increased from PND 50 to PND 110. At PND 50, an increased vascular area associated with higher VEGF expression was observed in the 250 BPA-treated rats. At PND 110, the vascular area was still increased, but VEGF expression was similar to that of control rats. The present results demonstrate that prenatal exposure to BPA alters the endocrine environment of the mammary gland and its angiogenic process. Increased angiogenesis and altered steroid hormone signals could explain the higher frequency of pre-neoplastic lesions found later in life.

This article is part of a Special Issue entitled 'Endocrine disruptors'.

1. Introduction

Epidemiologic studies suggest that the intrauterine hormonal milieu may predispose an individual to carcinogenesis. An increased risk of breast cancer has been noted with twin dizygotic female birth, a marker of high estrogen exposure [1], while preeclampsia, a marker of low estrogen exposure, is associated with lowered risk [2]. Currently, concern about the effects of prenatal estrogen exposure has focused on exposure to environmental estrogens, which may affect mammary gland development and/or enhance the risk of breast cancer later in life [3].

Over the last 60–70 years, a plethora of synthetic hormonally active chemicals, named endocrine disruptors, has been released into the environment. Among them, bisphenol A (BPA) is receiving increased attention because is a chemical used widely in the production of several resins (e.g., epoxy, polyester, polysulfone, and polyacrylate), polycarbonate plastics (food and drinking package, baby formula bottles), flame retardants, and dental sealants [reviewed in 4]. Research has demonstrated that the mammary gland is affected by BPA treatment. In mice, BPA perinatal exposure altered mammary gland maturation rates, delayed lumen formation, enhanced ductal growth, promoted a pregnancy-like state, enhanced responsiveness to secondary estrogenic exposures [5]; while in BPA perinatal exposed rats, an increased susceptibility to carcinogenesis (via increased number of hyperplastic ducts) was observed [6]. These findings suggest that developmental exposure to BPA may lead to a predisposition to breast cancer later in life [6,7].

Abbreviations: BPA, bisphenol A; CV, coefficients of variation; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; E_2, 17β-estradiol; EPA, Environmental Protection Agency; ER, estrogen receptor; FSH, follicle stimulating hormone; GD, gestation day; H&E, hematoxylin and eosin; IOD, integrated optical density; LH, luteinizing hormone; LOAEL, lowest observed adverse effect level; P_4, progesterone; PND, postnatal day; PR, progesterone receptor; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; SRC-3, steroid receptor co-activator 3; sc, subcutaneous; T_A, total area; V_A, vascular area; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.

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The increased incidence of hyperplastic ducts may be a consequence of misregulation in endocrine signaling pathways. Previously, we have demonstrated that prenatal exposure to BPA increases the sensitivity of the developing mammary gland to endogenous estrogen, thereby creating a permissive state that can lead to malignancy [5]. It is well established that steroid hormones can exert their effects by binding at specific receptors, and thereby modifying target gene expression. Steroid receptor-mediated gene regulation is complex, and depends on the recruitment of tissue-specific co-regulatory factors that differentially affect the interaction of receptors with their target genes. Progesterone receptor (PR) and estrogen receptor (ER)-regulated gene transcription are mediated through interactions with steroid receptor co-regulators, including the silencing mediators of retinoic acid and thyroid hormone receptor (SMRT) co-repressor, and proteins in the p160 steroid receptor co-activator (SRC) gene family [8]. The mammary gland, a target organ of ovarian steroids, expresses SRC-3 [9] and it was suggested that SRC-3 plays a role in PR- and/or ERα-mediated mammary gland development [10]. SMRT has been shown to play an active role in preventing tamoxifen from stimulating proliferation in breast cancer cells through repression of a subset of target genes involved in ERα function and cell proliferation [11]. Previous reports from our laboratory have demonstrated that postnatal BPA exposure affects the uterine responsiveness to steroid hormones in adulthood, possibly disrupting the transcription machinery's assembly of PR- and ER-dependent genes [12,13].

Moreover, inappropriate vessel growth underlies many pathological conditions, among them tumor growth and metastasis [14]. Numerous studies have shown that E_2 increases vascular endothelial growth factor (VEGF) expression, a key factor in blood vessels' growth [15,16]. VEGF partially regulates the angiogenic process, although angiogenesis is also affected by a diverse array of soluble mediators, matrix molecules, and accessory cells that function to orchestrate the growth, differentiation, and maturation of new capillaries [17]. Molecules that promote or inhibit angiogenesis can be produced by normal or tumor cells, can be mobilized from molecules in the extracellular matrix, or may be produced by cells recruited to the tumor side such as macrophages and mast cells [17]. Interestingly, BPA also increases VEGF expression in the female reproductive tract and the hypophysis [17,18].

We have previously demonstrated in rodent models [5,6] that increased susceptibility of the mammary gland to neoplastic transformation is anticipated by structural changes in the gland such as an increased proliferation/apoptosis ratio in both the epithelial and stromal compartments, a higher number of hyperplastic ducts and an increased number of mast cells surrounding the hyperplastic ducts. To better understand the processes involved in the aforementioned disruptions, in this study we assessed the effects of prenatal BPA exposure on mammary gland angiogenesis and steroid hormone pathways in virgin cycling rats.

2. Materials and methods

2.1. Animals

The experimental protocols were designed in accordance with the Guide for the Care and Use of Laboratory Animals issued by the U.S. National Academy of Sciences and approved by the ethical committee of the School of Biochemistry and Biological Sciences, Universidad Nacional del Litoral. Animals were treated humanely and with regard for alleviation of suffering. Sexually mature female rats (3 month-old) of a Wistar-derived strain bred at the Department of Human Physiology (School of Biochemistry and Biological Sciences, Santa Fe, Argentina) were used. Animals were maintained in a controlled environment ($22 \pm 2\,^{\circ}C$; 14 h of light from 0600 h to 2000 h) and had free access to pellet laboratory chow (Nutricion Animal, Rafaela, Argentina). The concentration of phytoestrogens in the diet was not evaluated; however, because food intake was equivalent for control and BPA-treated rats (unpublished data) we assumed that animals in the experimental and control groups were exposed to the same levels of phytoestrogens. To minimize other exposure to endocrine-disrupting chemicals, rats were housed in stainless steel cages with sterile pine wood shavings as bedding; tap water was supplied *ad libitum* in glass bottles with rubber stoppers.

2.2. Experimental procedures (Fig. 1)

Females in proestrous were caged overnight with males of proven fertility. The day that sperm was found in the vagina was designated day 1 of pregnancy [gestation day 1 (GD1)]. On GD8, corresponding to the beginning of organogenesis in the fetus, rats were weighed and implanted subcutaneously with a miniature osmotic pump (model 1002; Alza Corp., Palo Alto, CA, USA), which was prepared to deliver either 50% DMSO (vehicle-treated control; Sigma–Aldrich, Buenos Aires, Argentina), 25 μg BPA/kg/day (25 BPA; 99% purity Sigma–Aldrich), or 250 μg BPA/kg/day (250 BPA); 8–10 dams/group were included. BPA and DMSO were released continuously via the pump for 14 days (from GD8 to GD23) at a rate of 0.25 μl/h. After parturition (GD23), pups were weighed and sexed according to the anogenital distance and litters of eight pups (preferably four males and four females) were left with lactating mothers until weaning on postnatal day 21 (PND 21). The age at vaginal opening was not recorded in this experiment, but by PND 40 puberty onset had been attained by all female offspring. The effects of the treatment in female offspring at PND 50 and PND 110 were evaluated (one female per litter per time point in each group); the remaining females and males were assigned to other experiments. In spite of different administration routes, subcutaneous (sc) *versus* oral, we selected BPA doses taking as reference the lowest observed adverse effect level (LOAEL), and the safe dose established by the US Environmental Protection Agency (EPA). BPA doses used in this study, were lower than the LOAEL dose of 50 mg/kg/day; specifically, 25 BPA was equivalent to one-half of the safe dose established by the EPA (0.05 mg/kg/day) [19]. The other dose, however, was five-fold higher (250 BPA) than the safe dose.

2.3. Sample collection

To avoid estrous cycle related changes in the mammary growth and hormone serum levels, all samples were collected at diestrus I. Female rats from our colony exhibit a five-day estrous cycle, to determine the phases of each rat's estrous cycle, daily vaginal smears were evaluated for at least twelve days prior to sample collection [20]. Females were autopsied at the closest diestrus I to PND 50 (49–52) or PND 110 (110–114). At PND 50, we evaluated 9, 10 and 10 animals for DMSO, 25 BPA and 250 BPA, respectively. At PND 110, we evaluated 8, 9 and 10 animals for DMSO, 25 BPA and 250 BPA, respectively. Blood was collected and serum stored at $-80\,^{\circ}C$ until hormone assays were performed. One abdominal mammary gland from the 4th pair was obtained, fixed in 10% (v/v) buffered formalin, and embedded in paraffin and the contra lateral was kept for other studies.

2.4. Hormone assays

Serum levels of E_2 and progesterone (P_4) were determined by RIA using [2,4,6,7,16,17-^{3}H] E_2 and [1,2,6,7-^{3}H] P_4, respectively (Perkin–Elmer Life and Analytical Sciences Inc., Boston, MA, USA), as well as specific antibodies provided by Dr GD Niswender [21].